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New objective measurements of semen wave motion are associated with fertility in sheep

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Abstract. In sheep, wave motion in semen is currently used by AI centres to select ejaculates for insemination. Despite its low cost, convenience and established ability to predict fertility, the subjectivity of this assessment is a limiting factor for its applicability. The aims of the present study were to establish an objective method for the analysis of wave motion and to assess the associations of objective parameters with fertility after cervical insemination. Collective sperm motion in undiluted semen was observed by phase contrast microscopy at low magnification in a 100-µm deep glass chamber. Images of moving dark waves over a grey background were recorded and analysed by the optic flow method, producing several velocity-related parameters. Turbulence was assessed from the motion of fluorescent polystyrene beads. Among objective parameters, optical flow entropy and the average speed of beads were both able to discriminate ejaculates suitable for insemination. Two synthetic variables of optic flow and bead motion and a global objective variable were computed from linear combinations of individual parameters and compared with the subjective motion score for their predictive value. These were as efficient as the wave motion score for assessing fertility and can be proposed for the assessment of ram semen in routine AI procedures.

Additional keywords: fertility, mass motility, sperm, ram.

Introduction

The development of AI in French sheep farming dates back to the 1970s. In French sheep, AI is performed primarily by cervical insemination of fresh semen in synchronised oestrous ewes. The success of AI depends on various male and female factors, as well as factors related to insemination practice (David *et al.* 2008). Because a single ejaculate is used to perform several inseminations, it is important to establish criteria that will allow the successful selection of fertile semen. Various criteria expected to represent the sperm cell's ability to fertilise an oocyte have been proposed in the literature (Rodríguez-Martínez 2007). However, the relationship between most of these criteria and field fertility is still weak (Zhang *et al.* 1999; Rodríguez-Martínez 2003), suggesting that new powerful methods need to be developed (Petrunkina *et al.* 2007). The reason for the disconnection between *in vitro* evaluation of these sperm characteristics and fertility is because most of the

laboratory assays measure only part of the attribute that a sperm must have to ensure fertilisation (Mocé and Graham 2008). Thus, combining several criteria is recommended to provide a more reliable estimate of sperm fertility (Rodríguez-Martínez 2003). Nonetheless, combining various tests is not convenient for commercial AI centres. Actually, because most ewes in France are inseminated with fresh semen, the test used to select ejaculates for further insemination must be easy and fast to perform, as well as inexpensive (David *et al.* 2015). At present, a subjective scoring of wave motion (WM) or sperm mass motility (SMM) is currently used by French AI centres to select ejaculates. This scoring is based on observation of a semen droplet with a phase contrast microscope and the subjective assessment of rapid motion of black waves and whirlpools on a grey background. This type of semen evaluation is easy, cheap and fast to perform. In addition, in a study conducted with large populations, David *et al.* (2015) showed that WM was predictive

of the lambing rate. However, this evaluation of WM suffers from a major drawback: its subjectivity. Therefore, this scoring is not theoretically repeatable, which is inconsistent with the definition of a true test. Objective evaluation of individual sperm movement can be performed with computer-aided sperm analysis (CASA-Mot). The use of CASA-Mot allows detailed quantitative measurements of individual sperm cell motility from two-dimensional (2D) imaging, whereas WM is based on the observation of movement in three dimensions taking into consideration the collective movement of the sperm cells. This difference probably explains why CASA-Mot parameters are not good predictors of sperm fertility (Januskauskas *et al.* 1999; Gillan *et al.* 2008). Herein, we propose a new objective method for the assessment of WM in ram semen and evaluate its efficiency in the prediction of lambing rate after cervical insemination.

Materials and methods

Semen collection

The present study was based on a total of 531 ejaculates produced during spring 2014 (from 6 to 25 June) by 151 Lacaune rams belonging to one French AI centre located in the south-west of France (Roquefort). To synchronise semen production with the desired insemination period, rams received a photoperiodic treatment ~ 2 months before the beginning of the annual semen collection period at the centre (Chemineau *et al.* 1988). Semen collection was performed using an artificial vagina according to routine procedures as described by Baril (1993). Semen quality was assessed using three methods, namely (1) subjective assessment in semen drops, (2) objective assessment by optical flow in chamber and (3) objective assessment of bead motion.

Mobility assessment

Subjective assessment of WM in semen drops

A 5- μ L drop of raw semen was deposited on a prewarmed glass slide and observed using a phase contrast microscope ($\times 10$ magnification) equipped with a controlled heating stage (37°C). The focus was on the edge of the drop to visualise WM, the motion of black waves and whirlpools on a grey background. The speed of motion of these whirlpools was used as a subjective criterion to assess WM on a scale of 0 (no motion) to 5 (numerous rapid waves; Baril 1993), but implemented with 0.1-increments between scores of 4 and 5 to refine the assessment (David *et al.* 2015).

Objective assessment by optical flow of WM in chamber

A prewarmed 100- μ m deep glass chamber (Leja) was loaded with raw semen (30 μ L) and observed using a phase contrast microscope ($\times 10$ magnification) equipped with a controlled heating stage (37°C). A flow of short dark waves on a grey background could be observed. A sequence of 100 successive images (2560 pixels \times 2160 pixels) was recorded at a speed of 100 frames s^{-1} using a sCMOS pco.edge 5.5 camera (PCO AG) monitored using CamWare software (PCO). The velocity of the flow in the chamber was assessed using an accurate and fast

method derived from optical flow methods, without linearisation (Auroux and Fehrenbach 2011). A velocity field was obtained using the optical flow method and several criteria were derived from this, namely: (1) VAR, the variance of the velocity field (each component of the velocity considered independently), summed over the sequence; (2) DIV1, the L^1 norm of the divergence of the velocity field, averaged over the sequence; (3) ROT1, the L^1 norm of the rotational value of the velocity field, averaged over the sequence; (4) AUTO, the time constant corresponding to the best fitting of a decaying exponential with autocorrelation of the velocity field, computed with regard to the first velocity in the sequence; and (5) OFENT, entropy as a measure of the disorder of the velocity field. OFENT was computed as follows: the velocities were binned in a 20 pixels \times 20 pixels grid, and the proportion of vectors falling in bin j are denoted p_j . Then, the entropy of the vector field is defined by $H = -\sum_j p_j \ln(p_j)$ and is the time average of the entropy of the velocities at each instant.

Objective assessment of bead motion

Fluorescent Nile red beads (diameter 2 μ m; Spherotech) were added to raw semen. A prewarmed 100- μ m deep glass chamber was loaded with 30 μ L raw semen supplemented with beads and observed using a fluorescence microscope ($\times 4$ magnification) equipped with a controlled heating stage (37°C). A sequence of 100 successive images (2560 pixels \times 2160 pixels) was recorded at a rate of 100 frames s^{-1} . This sequence of images allowed the detection of white beads on a dark background, the trajectories of which are expected to be linked with the overall motion of spermatozoa in the semen sample.

The method to compute the parameters of the bead trajectories included three steps: (1) detection of the beads in each frame of the sequence; (2) tracking of each bead throughout the sequence to build its trajectory; and (3) computation of criteria for the trajectories of all beads. These criteria were average bead velocity (AVG), standard deviation of the average bead velocity (STD), the entropy of the instantaneous bead velocity (BENT) and BSCORE, a score based on AVG, STD and BENT from the learning of a non-linear regression curve using a support vector regression (SVR) approach (Drucker *et al.* 1997).

Synthetic variables

In addition to the five optical flow criteria (VAR, DIV1, ROT1, OFENT, AUTO) and the four bead speed criteria (AVG, STD, BENT and BSCORE), three synthetic variables (linear combination of the former criteria) were computed, namely: (1) OFS, an optical flow synthetic variable based on criteria measured with optical flow; (2) BS, a bead synthetic variable based on criteria measured with beads; and (3) GS, a global synthetic variable based on all new criteria together.

These synthetic variables were built as follows: reduced centred new criteria and a one-way interaction were included in the model as continuous variables, then significant factors were selected in a stepwise manner using the likelihood ratio test. The linear combination of significant new motility variables was then used to compute the synthetic variables. After

selection of criteria, the formulas used for the synthetic variables were as follow:

$$OFS = -0.02159 \times AUTO - 0.01239 \times OFENT - 0.00804 \times OFENT^2$$

$$BS = 0.04622 \times AVG - 0.02619 \times AVG^2 - 0.03837 \times STD + 0.01631 \times STD^2$$

$$GS = -0.01929 \times AUTO + 0.03144 \times AVG - 0.02204 \times AVG^2 - 0.03500 \times STD + 0.01135 \times STD^2$$

Cervical inseminations

Ejaculates with a subjective WM score >4 were used to produce insemination doses (480 ejaculates). Selected semen samples were then diluted in a skim milk extender (11.1 g per 100 mL water) supplemented with antibiotics at a final concentration of 1.0 or 1.4×10^9 cells mL^{-1} . Diluted semen was packaged in 0.25-mL straws and stored at 15°C until cervical insemination was performed within 6 h of collection. Before insemination, ewes received an oestrous synchronisation treatment with a fluorogestone acetate vaginal sponge (Sanofi Animal Health) inserted for 14 days, and an injection of pregnant mare's serum gonadotropin (PMSG) at withdrawal (Folligon; Sanofi Animal Health). Insemination was performed 55 h following sponge removal without detection of oestrus.

Analysis of fertility data

AI was defined as a success ($y = 1$) if lambing occurred 142–152 days after AI; otherwise, it was considered as failure ($y = 0$). Generalised linear models with logit link function were used to study the relationship between each motility criterion and the lambing rate. Other factors affecting AI success included in the model were selected in a stepwise manner, using nested models that were compared with each other with the likelihood ratio test. To avoid any assumption of the form of the relationship

between each criterion and fertility, motility criteria were included in the model as categorical variables in 10 classes (as for the subjective WM score). The values of each continuous new motility variable were grouped into classes according to the value limits and the sample size of each class. Once the classes had been defined, each motility criterion was included in the model one at a time leading to 10 different models.

Thirteen different criteria of sperm motility were then compared in the study (subjective WM score, nine new motility criteria and three synthetic variables). The quality of each criterion was evaluated by a visual inspection of changes in the estimated lambing rate with the criterion value, the goal being an increase of the probability success with the criterion value at least as important as the one observed with the subjective WM score. In addition, to compare the hypothesis of a linear increase in the lambing rate with motility criteria, all previous models were rerun with a motility criterion included as a continuous variable, with the Bayesian Information Criterion (BIC) used to compare the fit of each model to the data.

Results

WM of ram semen in drops and chambers

Representative images of ram semen observed with phase contrast microscopy in a drop or in the 100- μm deep chamber are shown in Fig. 1. The observation of the edge of the drop showed dark waves and whirlwinds actively appearing and disappearing on a light background (Fig. 1a). The speed of the spinning of those whirlwinds was used as the main criterion to generate a subjective WM score. A representative sample movie with a WM score equal to 5 is available as Clip S1, available as Supplementary Material to this paper.

The observation of WM in 100 μm deep chambers revealed a different pattern of dark waves compared with the waves in semen drop (Fig. 1b; Clip S2). The use of calibrated chambers with a standardised depth allowed the production of more standardised images better suited for automatic computerised analysis. Optical flow analysis was performed on these images and allowed the computing of motion vectors (Fig. 1c).

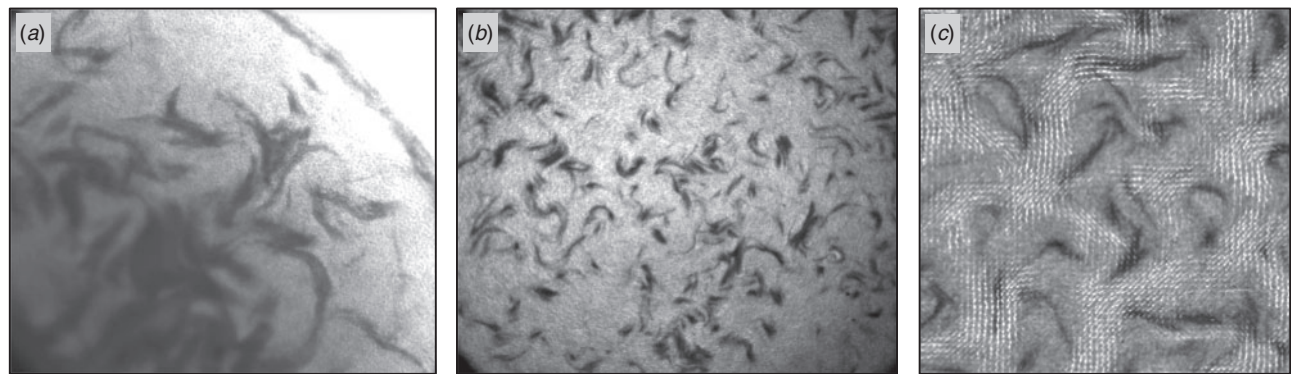


Fig. 1. Phase contrast imaging of wave motion in ram semen. (a) A drop of raw ram semen was deposited onto a glass slide and the edge of the drop was observed using phase contrast microscopy. (b) A 100- μm deep glass chamber was filled with semen and observed using phase contrast microscopy. (c) Optic flow analysis of phase contrast images of semen in the chamber allowed the detection of velocity fields (vectors shown by white arrows).

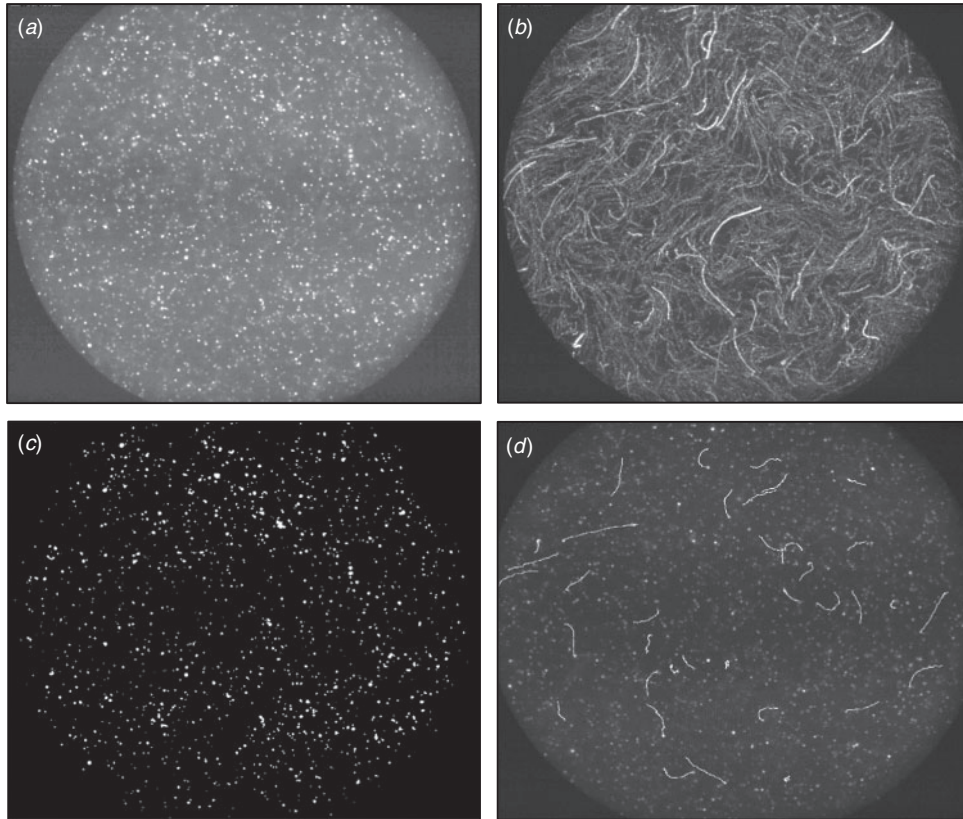


Fig. 2. Imaging of bead motion in raw semen. (a) Single image of semen sample mixed with beads and observed using fluorescence microscopy. (b) Stack of the images from a single sample showing the trajectories observed during one assessment. (c) Image of beads detected using the detection routine. (d) Examples of computed trajectories providing trajectory parameters.

Bead motion in raw semen

Individual bead trajectories could be observed correctly using fluorescence microscopy (Fig. 2; Clip S3). The image analysis procedure was able to detect the beads and reconstruct their trajectories. In all, 1300 beads were detected and an average of 200 trajectories was computed for each sample. The selection of trajectories for subsequent computing of parameters was stringent because only trajectories with at least 100 images and a stable speed were kept.

Objective assessment of WM

The descriptive statistics of the criteria of mobility and their correlations are given in Tables 1 and 2. Bead movements criteria (AVG, STD, BENT) were strongly correlated with each other (correlation $r > 0.80$) and showed a similar relationship with the WM score (correlation > 0.4).

Three of the optical flow (OF) criteria also showed a high correlation with each other (DIV1, ROT1 and OFENT; correlation > 0.72) and were poorly correlated with the other OF criterion (AUTO; correlation < 0.26).

The OFENT and AUTO parameters were the two OF parameters selected by the step-by-step model to create the OF synthetic variable. OFENT and AUTO showed a negative

non-linear relationship with the WM score over the range 1–5 (Fig. 3).

The AVG and STD parameters describing the speed of the beads showed a positive non-linear relationship with the WM score over the range 1–5 (Fig. 4). AVG and STD were significantly higher when the WM score was > 4 .

Cohen's κ coefficient was calculated to assess the concordance of the ejaculate selection based on the AVG, STD and OFENT parameters or the subjective WM score. The thresholds values for AVG, STD and OFENT were 1, 0.7 and -2 respectively, and gave similar κ values of 0.49, 0.50 and 0.45 respectively. Regarding the accuracy of detection of bad-quality ejaculates and the ability to discard semen with potentially low fertility, the AVG parameter showed the highest sensitivity and specificity, with values of 88% and 77% respectively.

Prediction of fertility with objective methods of WM assessment

Once the link between ejaculate and AI results had been established (ewes in the French national performance recording database), a dataset of 5479 AIs corresponding to 215 ejaculates was used for the analysis. The observed lambing rate was 66.1%, which is in accordance with the lambing rate reported previously

Table 1. Descriptive statistics of sperm criteria

Data are given as the mean [minimum, maximum]. WMS, wave motion score; VAR, variance of the velocity field, summed over the sequence; DIV1, L¹ norm of the divergence of the velocity field, averaged over the sequence; ROT1, L¹ norm of the rotational value of the velocity field, averaged over the sequence; AUTO, time constant corresponding to the best fitting of a decaying exponential with autocorrelation of the velocity field, computed with regard to the first velocity in the sequence; OFENT, entropy as a measure of the disorder of the velocity field; AVG, average bead velocity; STD, standard deviation of AVG; BENT, entropy of the instantaneous bead velocity; BSCORE, score based on AVG, STD and BENT from the learning of a non-linear regression curve using a support vector regression approach; OFS, optical flow synthetic variable based on criteria measured with optical flow; BS, bead synthetic variable based on criteria measured with beads; GS, global synthetic variable based on all new criteria together

Method	Criterion	All ejaculates (n = 531)	Ejaculates with known fertility(n = 243)
Subjective	WMS	4.35 [2, 5]	4.53 [4, 5]
Optical flow	VAR	15 840 [46, 53 239]	16 001 [46, 53 239]
	DIV1	1.64 [0.51, 3.55]	1.66 [0.65, 2.72]
	ROT1	2.15 [0.57, 4.31]	2.20 [0.67, 3.53]
	OFENT	-2.78 [-4.16, 0.00]	-2.87 [-3.95, 0.00]
	AUTO	92.34 [14.15, 1265.40]	79.75 [14.15, 376.30]
Beads	AVG	1.35 [0.24, 4.98]**	1.37 [0.24, 3.28]*
	STD	0.92 [0.24, 1.63]**	0.95 [0.29, 1.62]*
	BENT	2.49 [0.67, 3.77]**	2.55 [0.67, 3.33]*
	BSCORE	4.01 [3.48, 4.68]**	4.06 [3.48, 4.68]*
Synthetic variables	OFS	-0.01 [-0.22, 0.05]	-0.01 [-0.28, 0.03]
	BS	-0.01 [-0.17, 0.31]**	-0.01 [-0.12, 0.10]*
	GS	-0.02 [-0.41, -0.35]**	-0.01 [-0.13, 0.10]*

Table 2. Spearman correlation coefficients between sperm criteria

WMS, wave motion score; VAR, variance of the velocity field, summed over the sequence; DIV1, L¹ norm of the divergence of the velocity field, averaged over the sequence; ROT1, L¹ norm of the rotational value of the velocity field, averaged over the sequence; AUTO, time constant corresponding to the best fitting of a decaying exponential with autocorrelation of the velocity field, computed with regard to the first velocity in the sequence; OFENT, entropy as a measure of the disorder of the velocity field; AVG, average bead velocity; STD, standard deviation of AVG; BENT, entropy of the instantaneous bead velocity; BSCORE, score based on AVG, STD and BENT from the learning of a non-linear regression curve using a support vector regression approach; OFS, optical flow synthetic variable based on criteria measured with optical flow; BS, bead synthetic variable based on criteria measured with beads; GS, global synthetic variable based on all new criteria together

		Beads				Optical flow				Synthetic variables			Subjective mobility
		STD	BENT	BSCORE	VAR	DIV1	ROT1	OFENT	AUTO	BS	OFS	GS	WMS
Beads	AVG	0.80	0.89	0.01	0.15	0.45	0.52	−0.48	−0.25	0.60	0.52	0.50	0.43
	STD		0.96	0.11	0.10	0.44	0.50	−0.44	−0.22	0.31	0.49	0.26	0.42
	BENT			0.07	0.11	0.44	0.51	−0.46	−0.23	0.45	0.51	0.36	0.44
	BSCORE				−0.25	0.06	0.12	−0.15	−0.01	0.15	0.13	0.13	0.11
Optical flow	VAR					0.19	0.22	−0.22	−0.28	0.08	0.32	0.18	0.13
	DIV1						0.93	−0.72	−0.26	0.25	0.70	0.29	0.44
	ROT1							−0.88	−0.22	0.30	0.78	0.31	0.49
	OFENT								0.03	−0.30	−0.71	−0.20	−0.46
Synthetic variables	AUTO									−0.15	−0.66	−0.59	−0.23
	BS										0.32	0.81	0.27
	OFS											0.58	0.52
	GS												0.28

for this breed (66.7%; David *et al.* 2015). Changes in the lambing rate with WM estimated with the generalised linear model (least square means) are shown in Fig. 5. An increase of 13 points in the lambing rate was observed from the lowest to the highest value of the WM score (range 4–5). The estimated

changes in the lambing rate with the synthetic criteria were superimposed with the WM score curve (Fig. 5). The OFS, BS and GS variables all showed a significant correlation with fertility, with the best result being between the GS variable and fertility.

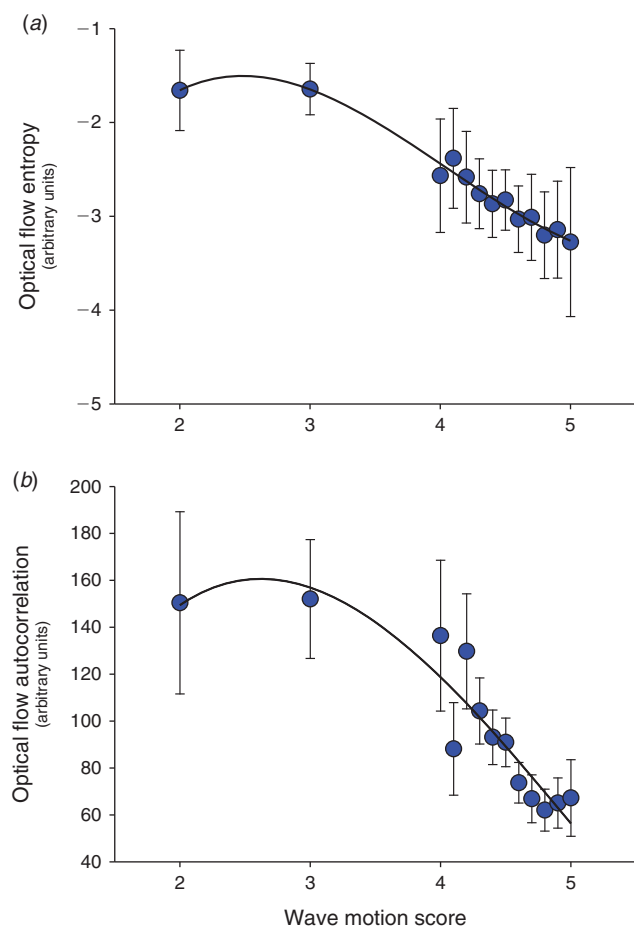


Fig. 3. Relationship between subjective wave motion score and optical flow parameters, namely (a) optical flow entropy and (b) optical flow autocorrelation. Data are expressed as the mean \pm s.e.m. ($n = 531$ ejaculates).

Discussion

WM scoring is based on observations using phase contrast microscopy at low magnification of a drop of semen deposited on a glass slide. A survey we performed on several sheep AI centre operators revealed that this WM scoring, although resulting from a complex combination of subjective parameters, primarily consists of the assessment of the global turbulence of the sample and the speed of rotation of whirlwinds (I. David, unpubl. data). Therefore, an objective method had to be chosen for its ability to assess the turbulence of a fluid with beads.

Because the features of such waves may be affected by the operator-dependent shape and thickness of the drop, it was decided to use a more standardised procedure of semen observation. Glass chambers were chosen with a standard inner dimension (100 μ m deep) allowing the three-dimensional collective movement of spermatozoa leading to WM while ensuring repeatable conditions of assessment.

The analysis of WM was then performed following two complementary strategies. First, sperm movement was assessed

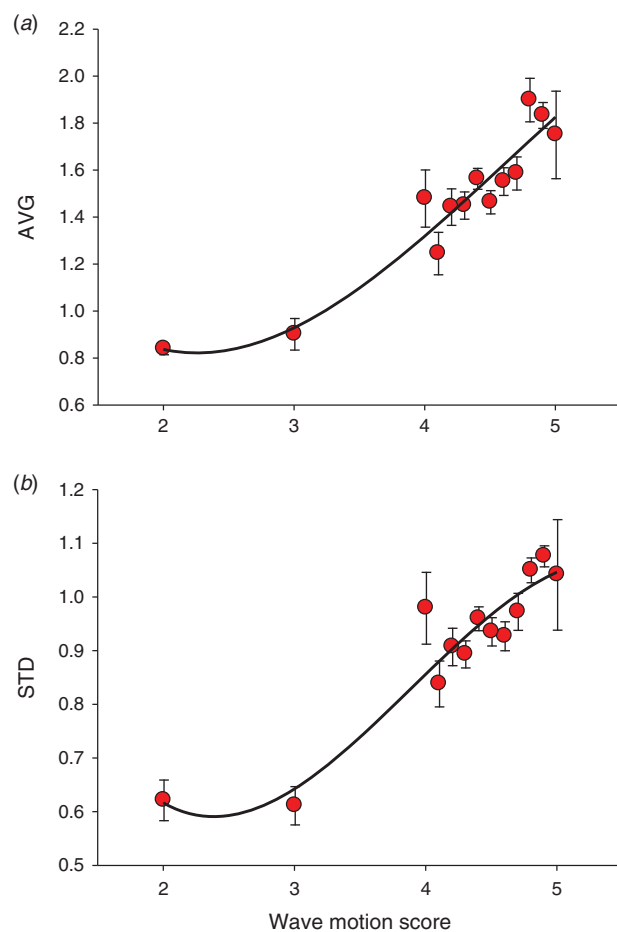


Fig. 4. Relationship between subjective wave motion score and bead motion parameters, namely (a) the average speed of beads (AVG) and (b) the standard deviation of AVG. Data are expressed as the mean \pm s.e.m. ($n = 498$ ejaculates).

directly by observation using phase contrast microscopy at low magnification. The WM observed in the glass chamber was different from that observed from drops by exhibiting a laminated flow structure (Creppy *et al.* 2015). This type of image, made up of dark waves over a grey background, could be assessed by an image analysis OF method using a global comparison of successive images to compute translational and rotational motion parameters (Auroux and Fehrenbach 2011). A second and complementary strategy was not to analyse the sperm cells, but to measure the turbulence of the semen by loading the sample with 2- μ m diameter beads and assessing the bead trajectories and their properties. The use of fluorescent beads resulted in the production of standardised images with white spots over a dark background.

The main practical purpose of the WM score in AI is to detect low-quality ejaculates by eliminating samples with a score < 4 . Individual objective parameters, such as the OFENT and AVG, showed similar and high κ coefficients, as well as elevated specificity and sensitivity, suggesting that both methods can be used to discriminate low-quality ejaculates.

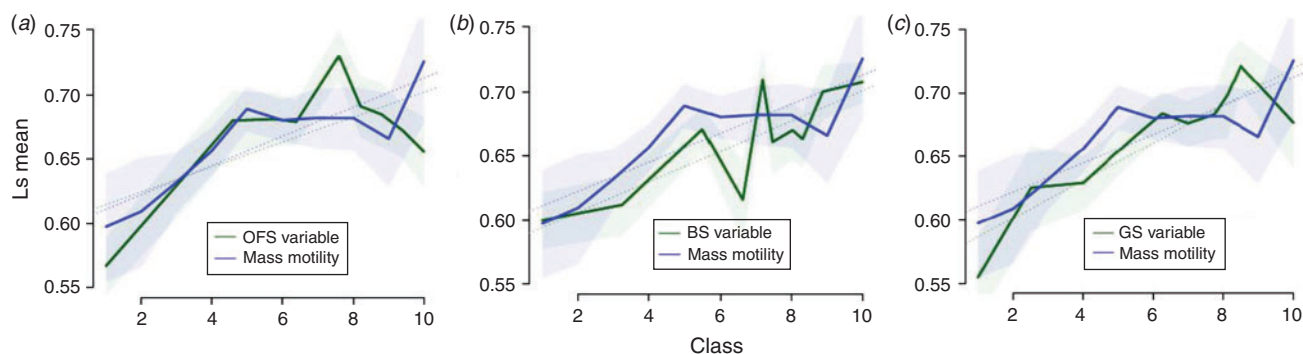


Fig. 5. Relationship between fertility after AI and semen assessments. Ram ejaculates were assessed for subjective wave motion score, optical flow and bead motion ($n = 215$). The wave motion score and the optical flow synthetic (OFS) variable (a), bead synthetic (BS) variable (b) and global synthetic (GS) variable (c) data were grouped in 10 classes and linked to fertility in a logistic regression model ($n = 5479$ AI cycles). Fertility is expressed as the least squares (LS) mean of the lambing rate computed from logistic regression analysis. The shaded areas indicate the standard deviation of the means.

The WM score was shown previously to be positively linked with fertility in a large-scale study including more than 800 000 inseminations in sheep (David *et al.* 2015). Despite many fewer inseminations (5000 ewes), the results from the present study were consistent with that report and confirmed the link between WM and fertility after AI in sheep.

Based on a selection of parameters computed from the objective methods and significantly correlated with the WM score, OFS and BS variables were calculated and compared with fertility following AI. Both were positively associated with fertility, but the GS variable, a combination of the OFS and BS variables, showed the best link with fertility, similar to the WM score. However, it should be noted that coefficients of the synthetic variable were estimated and validated on the same dataset, leading to conclusions that are dataset specific. To make a valid generalisation of the results, application to another dataset or cross-validation should be performed. These findings suggest that both sperm motion and fluid turbulence are involved in the link with fertility.

Given the generic principles of these objective methods, tools of WM assessment could be developed for a range of species exhibiting high sperm concentrations and WM, such as goat, cattle and chicken.

Data accessibility

Data supporting this work are available on the Figshare website in the project entitled “New objective measurements of semen wave motion are associated with fertility in sheep” (<https://doi.org/10.6084/m9.figshare.5756448.v1>, accessed 5 January 2018).

Conflicts of interest

The authors declare no conflicts of interest.

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